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Stage-specific Expression of Ankyrin and SOCS Box Protein-4 (Asb-4) during Spermatogenesis

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Members of the large family of Asb proteins are ubiquitously expressed in mammalian tissues; however, the roles of individual Asb and their function in the developmental testes have not been reported. In this report, we isolated a murine *Asb4* from mouse testis. Northern blot analysis revealed that *mAsb-4* was expressed only in testes and produced in a stage-specific manner during spermatogenesis. It was expressed in murine testes beginning in the fourth week after birth and extending into adulthood. Pachytene spermatocytes had the highest level of expression. Interestingly, the human homologue of *mAsb-4, ASB-4* (*hASB-4*) was also expressed in human testis. These results suggest that ASB-4 plays pivotal roles in mammalian testis development and spermatogenesis.

Keywords: Ankyrin-Repeat; Asb-4; SOCS Box; Spermatogenesis.

Introduction

The suppressor of cytokine signaling (SOCS) family of proteins contributes to the negative regulation of cytokine signaling such as seen in the JAK-STAT pathway (Hilton et al., 1998; Kisseleva et al., 2002). Previous reports suggest that the SOCS box plays a role in ubiquitin-mediated proteasomal degradation via interaction with the elongin B and elongin C complex (Chung et al., 2005; Kamura et al., 1998; Krebs and Hilton, 2000).

Ankyrin repeats occur in molecules with a wide variety of functions including receptors, cell-cycle regulators, membrane skeletal proteins, secreted proteins, tumor suppressors, and transcription factors (Breeden and Nasmyth, 1987; Kile et al., 2000; Zhang et al., 1999).

Spermatogenesis is a cyclic developmental process by which spermatogonial stem cells generate mature spermatozoa via a unique genetic and molecular program (Kho and Inaba, 2004; Rhee and Wolgemuth, 2002). Many germ cell-specific genes are involved in this developmental process, e.g., *mAsb-17*, as discussed earlier (Kim et al., 2004). The mechanism that regulates the genes involved is elaborate, and understanding of this finely tuned mechanism remains elusive.

Following on from our study of mAsb-17, we report that a member of the ankyrin repeat-containing SOCS box protein family, Asb-4, shows unique tissue- and stage-specific expression.

Materials and Methods

Mice gonads and testes Male ICR mice (5 d, 2, 4, 6, and 10 weeks old) and pregnant female ICR mice (12.5 dpc and 15.5 dpc) were purchased from Daehan Biolink Co. Ltd. (Korea). The procedures for animal care and management was followed by SOP of Hanyang University, College of Medicine, Animal Care and Use Committee.

Collecting gonadal germ cells To collect germ cells, gonads were dissected and removed from 12.5 dpc and 15.5 dpc embryos with fine hypodermic needles and forceps. They were washed briefly in calcium-and magnesium-free phosphate-buffered solu-

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tion and incubated in an EDTA solution (20 mg EDTA, 800 mg NaCl, 20 mg KCl, 115 mg Na_2HPO_4, 20 mg KH_2PO_4 in 100-ml distilled water) for 20 min at room temperature. They were then washed in Dulbecco's modified Eagle's medium (DMEM) [10% fetal bovine serum (FBS)] and gently disrupted using a fine needle. The released germ cells were collected with a fine-drawn Pasteur pipette.

Isolation of spermatogenic cell populations Mixed populations of spermatogenic cells were obtained from testes of 10-weeks-old male mice using the collagenase dissociation method (Bellve et al., 1977). Purified populations of spermatogenic cells were isolated following collagenase treatment of testes and trypsin digestion of isolated seminiferous tubules using unit gravity sedimentation in a bovine serum albumin gradient (Bellve et al., 1977; Romrell et al., 1976). Tubes containing each cell population (pachytene spermatocyte (PS), > 90% pure; round spermatid (RS), > 90% pure; and condensing spermatid (CS)-residual body mixture) were selected by phase contrast microscopy.

Isolation and analysis of human testicular tissue from male infertility patients Testicular tissues were obtained from male infertility patients using the multiple testicular sperm extraction (TESE)-ICSI program. When sperm or round spermatids were absent from dissected samples, the remaining testicular tissues were donated for experiment with informed consent (Lee et al., 2006). This study was approved by the Institutional Review Board of the CHA General Hospital (Korea). To identify the existence of germ cells in the human testis samples from the male infertility patients, we investigated the expression of the specific marker genes for transition protein (TP)-1 and protamine. After confirming the presence of germ cells, RT–PCR analysis of *hASB-4* was performed.

STO and mouse ESC Culture Mouse STO fibroblast feeder cells [CRL-1503; American Type Culture Collection (ATCC), USA] were grown in DMEM (GIBCO/BRL, USA) supplemented with 10% FBS (HyClone, USA) and 1% penicillin-streptomycin (GIBCO/BRL, USA). These cells were mitotically inactivated with 10 μg/ml mitomycin-C (Sigma, USA) for 1.5 h. HS-3 mouse ESC were grown under standard conditions. Both cell types were grown in 5% CO_2, 95% air and were routinely passaged every 4–5 days.

RT-PCR for expression analysis Total cell RNA was isolated from mouse gonads, testes, STO, and mESCs using TRIzol (Invitrogen, USA). Whole mouse testes were obtained from mice at 5 days, and 2, 4, 6, and 10 weeks. PS, RS, and CS were separated from whole testes of 10-weeks-old mice by STA-PUT. One microgram of total RNA from each population was treated with DNase I (Sigma, USA). cDNA synthesis from total RNA was performed according to the protocol of the Super Script Preamplification system (Invitrogen, USA). After incubation of 1 μg total RNA with 0.5 μg oligo $(dT)_{12\text{-}18}$ primer at 70°C for 10 min, the reaction was carried out in the 5X first strand buffer (containing 250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$) from the kit, 10 mM DTT (dithiothreitol) and 0.5 mM dNTPs in a final volume of 20 μl at 42°C for 2 min. Then SuperScript™ II (Invitrogen, USA) was added and incubated for 50 min at 42°C. After the reaction, the enzyme was denatured at 70°C for 15 min. Primers used for amplification of *mAsb-4* were 5′-CAAAGCT-CAACTGCTACTCC-3′ (forward) and 5′-CAG-CTGGTAGCA-GATCTCA-3′ (reverse; product 539 bp) (GenBank accession number: *mAsb-4*; BC046819). Amplification was achieved by 28 cycles of 94°C for 35 s, 55°C for 40 s, and 72°C for 45 s followed by a final incubation for 7 min at 72°C. As a loading control, the same amounts of cDNA templates were used to amplify *G3PDH*. The oligonucleotide primers used for amplify-cation of *mG3PDH* were 5′-ACTGGTGCTGCC-AAGGCT-GT-3′ (forward) and 5′-CGGCATCGAAGGTGG-AAGAG-3′ (reverse; product 262 bp).

To identify the expression of specific marker genes in the testes from male infertility patients, we investigated the expression of the TP-1 and protamine genes. Primers used for amplification were 5′-gtcaagagag gtggcagcaa -3′ (forward) and 5′-tcacaagtgg gagcggtaat -3′ (reverse; product 104 bp) (GenBank accession number: NM_003284) and 5′-cgaggtgtac aggcagcagt-3′ (forward) and 5′- gcctccttcg agagcagtgt-3′ (reverse: product 145 bp) (GenBank accession number: NM_002761), respectively. Amplification was achieved by 30 cycles of 94°C for 35 s, 55°C for 30 s, and 72°C for 30 s followed by a final incubation for 5 min at 72°C.

Northern blot analysis Expression of *mAsb-4* mRNA from mouse tissue was analyzed by Northern blot analysis. Mouse multiple tissue blot was purchased from Seegene (Seegene, Korea). For the stage-specific mouse blot, RNA samples (20 μg each) of STO, 12.5 dpc, 15.5 dpc, 5 days, 2 weeks, 4 weeks, 6 weeks, 10 weeks, pachytene spermatocyte, round spermatid, condensing spermatid and mESCs were electrophoresed and blotted onto Hybond™-N+ membranes (Amersham Biosciences, UK). The immobilized nucleic acids were hybridized with the *mAsb-4* probe labeled by random priming with [α-^{32}P] dCTP (Amersham Biosciences, UK). Hybridization was performed in a bag containing ExpressHyb™ Hybridization solution (Clontech, USA) at 68°C overnight. The hybridized membrane was washed at room temperature in 2× SSC and 0.1% SDS, then in 0.1× SSC and 0.1% SDS, and exposed to Kodak X-ray film with an intensifying screen for 5 h at −70°C.

Results

Alignment of amino acid sequences for mouse, and human Asb-4 Mouse Asb-4 has an open reading frame of 1278 bp encoding a putative protein of 426 amino acids. Figure 1A shows the amino acid sequences of Asb-4 family members searched in GenBank aligned using the MegAlign with DNASTAR program. The amino acid sequence of

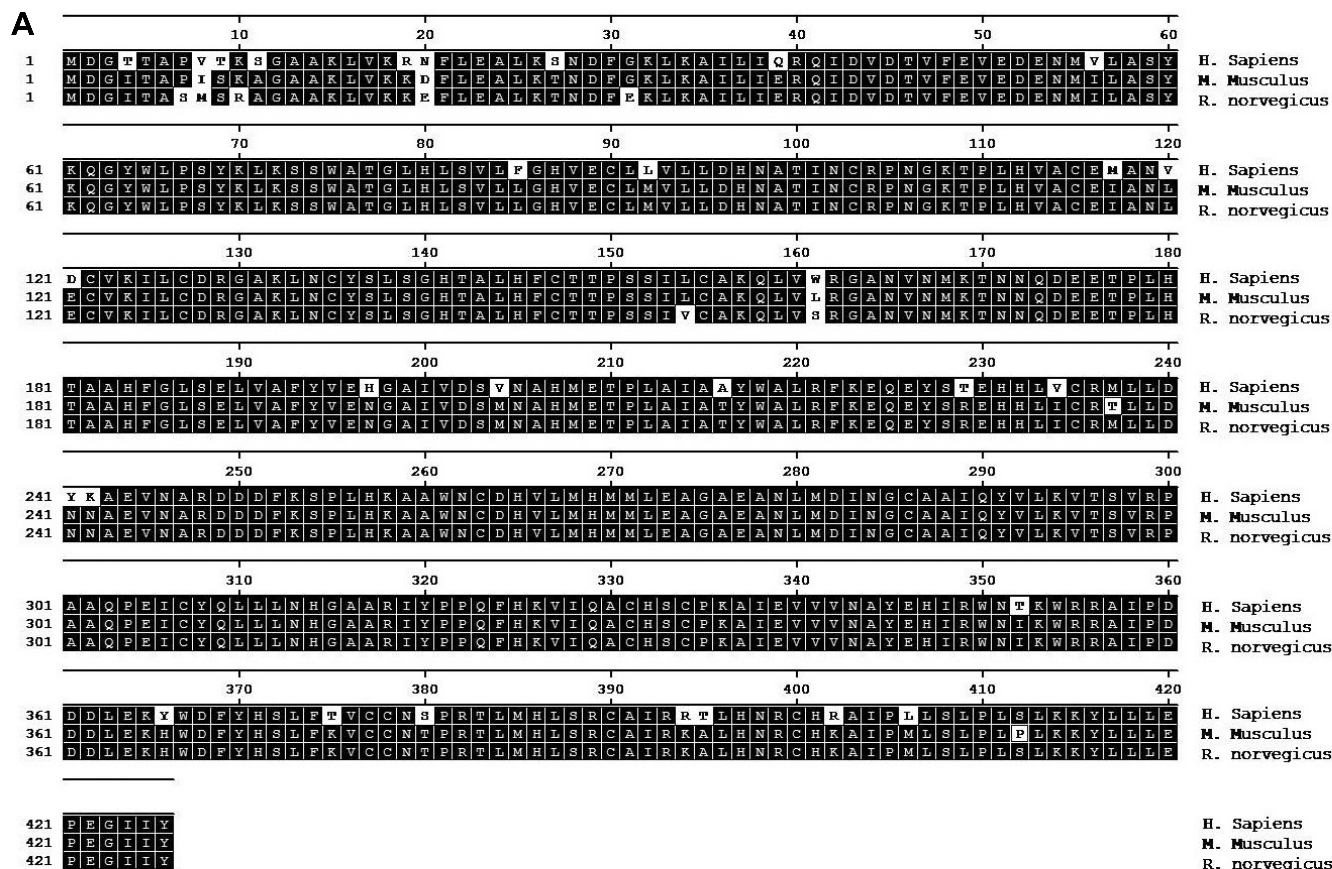

Fig. 1. A. Alignment of amino acid sequences of mouse Asb-4 (NP_075535), human ASB-4 (NP_057200), Rat Asb-4 (NP_001019489) using MegAlign with DNASTAR program. **B.** Domain of mouse Asb-4 contains six ankyrin repeats (ANK: amino acids 74-103, 106-135, 139-168, 174-203, 207-247, and 251-280) and one SOCS box (371-423).

mAsb-4 has 92% and 97% identity to that of humans and rat respectively (Table 1). SMART (http://smart.embl-heidelberg.de) showed that the deduced amino acid sequence of the mAsb-4 contains six ankyrin repeat domains (74–103, 106–135, 139–168, 174–203, 207–247 and 251–280 amino acids, respectively), and one SOCS box domain between amino acids 371 and 423 (Fig. 1B).

Table 1. The nucleotide and amino sequences of mAsb-4 and other members of the Asb-4 family.

Mouse (mAsb-4)	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
Human	85	92
Rat	93	97

Expression of *Asb-4* in mouse and human tissue To characterize the expression of *mAsb-4* in various tissues, we performed a Northern blot analysis (Fig. 2A). Total RNA was prepared from mouse brain, heart, lung, liver, spleen, kidney, stomach, small intestine, skeletal muscle, thymus, testis, non-pregnant uterus, and placenta. Strikingly, *mAsb-4* expression was seen only in the testis. We performed RT-PCR using a pair of primers designed from the sequence of a cDNA library clone (GenBank accession number: BC046819; Fig. 2B) to further investigate expression in various spermatogenic cells. PCR showed that *mAsb-4* was expressed after the fourth week of mouse testis development (Fig. 2B; lanes 4–6). In addition, Northern blot analysis revealed that the *mAsb-4* transcript of about 3 kb length was most strongly expressed in pachytene spermatocytes and was down regulated during further progression into condensing spermatids (Fig. 2C).

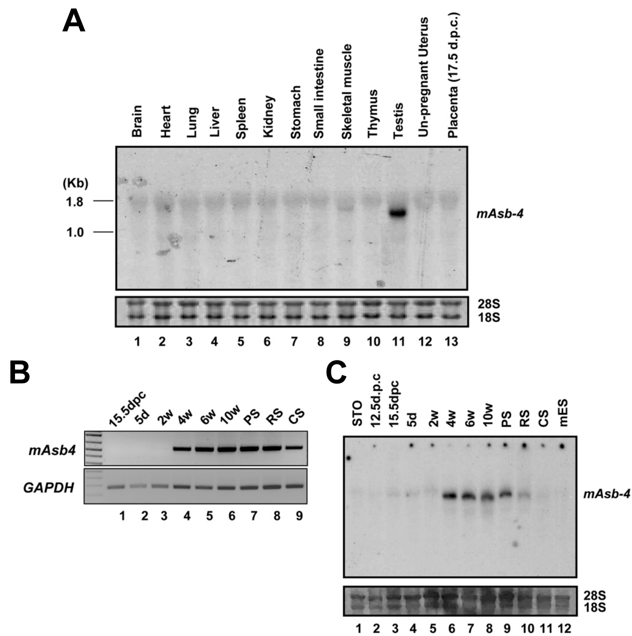

Fig. 2. A. Expression of *mAsb-4* mRNA in mouse organs. Total RNA was prepared from mouse brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), spleen (lane 5), kidney (line 6), stomach (lane 7), small intestine (lane 8), skeletal muscle (lane 9), thymus (lane 10), testis (lane 11), non-pregnant uterus (lane 12), and placenta (lane 13). Expression was seen only in testis. 28s/18s RNAs were used as loading controls. **B.** *mAsb-4* expression during mouse testis development and spermatogenesis, shown by RT-PCR. Lane 1 is a testicular sample from 15.5 dpc fetal gonads and lanes 2–6 are testicular samples from 5-day-old, 2-, 4-, 6-, and 10-weeks-old mice. In addition, cDNA samples from pachytene spermatocytes (PS), round spermatids (RS), and condensing spermatids (CS) were used. To assess RNA integrity, a *GAPDH* control was included. **C.** Northern blot hybridization of *mAsb-4* mRNA from 12.5 and 15.5 dpc fetal gonads (lanes 2, 3), 5-day-old, 2-, 4-, 6-, and 10-weeks-old mouse testis, pachytene spermatocytes (PS), round spermatids (RS), and condensing spermatids (CS) during spermatogenesis. mRNA from mouse embryonic stem cells (mESC) and STO cells was used as control. 28s/18s RNAs were served as loading controls.

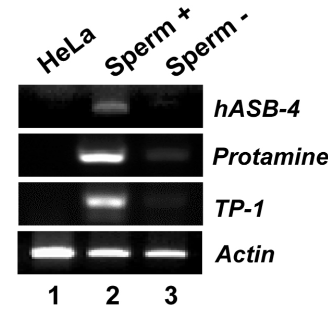

Fig. 3. RT-PCR analysis of *hASB-4* and germ-cell-specific marker genes in testicular tissues isolated from male infertility patients. Expression of TP-1 and protamine genes indicates the presence of germ cells. *hASB-4* was uniquely expressed in germ cell-containing samples. HeLa cells served as negative control. To determine RNA integrity, an actin positive control was included.

As there were no earlier reports of expression of *hASB-4* in human testicular samples we obtained human testicular samples from male infertility patients and tested for the presence of germ cells using the germ cell marker genes for TP-1 and protamine, and. examined the expression of *hASB-4* in the same samples. Interestingly, *hASB-4* was expressed only in the samples containing germ cells (Fig. 3; lane 2).

Discussion

Cytokines regulate fundamental biological processes such as immunity, wound healing, and hematopoiesis by interacting with receptors on cell surfaces (Ochoa et al., 2000). Negative feedback is another important mechanism regulating signal transduction by a wide variety of extracellular signals. Suppressors of cytokine signaling (SOCS) proteins are known as negative regulators of signal transduction. It is assumed that the SOCS box plays a role as a negative regulator in signal transduction, perhaps by inhibiting kinase activity. Another possibility is that the SOCS box acts as an adaptor module or regulates aspects of protein behavior such as intracellular location or stability (Hilton et al., 1998).

Very little is known about the ASB subfamily. Testes lacking *Asb-1* show a reduction of spermatogenesis with less complete filling of the seminiferous tubules (Kile et al., 2000; 2001). Exogenous expression of *Asb-2* in myeloid leukemia cells resulted in growth inhibition and chromatin condensation (Guibal et al., 2002). *Asb-6* interacts with APS to enable recruitment of elongins B and C to the insulin receptor-signaling complex in adipocytes. In sum, it is thought that ASB family members have pleotropic functions through protein-protein interactions.

In addition to expression of *mAsb-17* as we described earlier, *mAsb-4* expression has been reported to be restricted to mouse testis (lanes 8, 11); we confirmed this result here (Fig. 2A). In addition, we found that *mAsb-4* expression differed noticeably during spermatogenesis. Mice attain puberty at 5–6 weeks. The testes descend into the scrotum and produce sperm after the fifth week and male mice develop sexual maturity between 6 and 8 weeks of age. The pachytene phase of meiosis has a fixed duration of over a week for each particular species in pachytene spermatocytes (meiotic cells). Genetic recombination also occurs during this period. After the second meiosis, round spermatids (early haploid cells) undergo a series of shape changes, and condensing spermatids (late

haploid cells) also undergo marked transformations ultimately producing species-specifically shaped spermatozoa, in a process referred to as spermiogenesis. Global inhibition of the initiation of mRNA translation occurs in pachytene spermatocytes and round spermatids (Park et al., 2001). *mAsb-4* begins to be expressed near puberty and is continuously expressed thereafter. In spermatogenic cells, pachytene spermatocytes show the strongest expression of *mAsb-4*, while in the next stage of spermatogenic cells, round spermatids have weaker expression levels (Fig. 2C). Human testicular samples containing developing germ cells also showed expression of human *ASB-4*. Our findings imply that this unique gene is closely associated with testes development and spermatogenesis.

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